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Award Number: DAMD17-99-2-9050

TITLE: Synthesis of Carbocyclic 3-Deazaadenosine Analogs as Potential Agents Against Poxvirus

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REPORT DATE: October 2000

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2000	Final (1 Oct 99 – 30 Sep 00)

4. TITLE AND SUBTITLE

Synthesis of Carbocyclic 3-Deazaadenosine Analogs as Potential Agents Against Poxvirus

5. FUNDING NUMBERS

DAMD17-99-2-9050

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Our goal has been the development of new agents against the poxvirus models of variola virus, directing our efforts toward new carbocyclic 3-deazaadenosine (CADO) analogs and adenosine N^1-oxide analogs. CADO and some of its analogs (such as the 5'-carboxamide congener) had previously been shown to be active against a number of viruses including vaccinia virus. Based upon the knowledge of CADO and the metabolism of nucleosides in general, we targeted three distinct classes of potentially enhanced agents: (1) CADO analogs with selected alterations at C-5'; (2) similar analogs based on 4'-thio-3-deazaadenosine; and (3) CADO prodrugs resulting from 5'-O-acylation. In the term of this project, we were successful in preparing and delivering more CADO and one of our CADO congeners, the 5'-O-benzoate of CADO.

We also supplied Dr. Huggins with adenosine N^1-oxide and 22 adenosine N^1-oxide and 1-benzyloxyadenosines analogs. Because of the significant activities against vaccinia virus that they had previously shown, Dr. Huggins was interested in investigating their activities against variola and other poxviruses.

14. SUBJECT TERMS

carbocyclic 3-deazaadenosine, CADO, CADO prodrugs, CADO analogs, adenosine N^1-oxide, 1-benzyloxyadenosines, poxvirus, variola virus, vaccinia virus, nucleoside analogs

15. NUMBER OF PAGES

16

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Table of Contents

Introduction

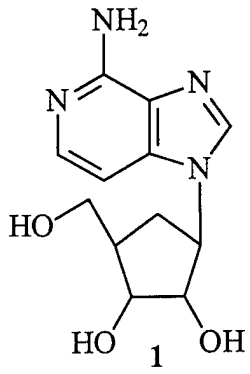
The goal for this contract has been the development of new potential agents against the poxvirus models of variola virus, with the synthesis of new carbocyclic 3-deazaadenosine (CADO) analogs as our primary focus. Recent studies had shown that carbocyclic 3-deazaadenosine and analogs (such as the 5'-carboxamide congener) were active against a number of viruses including vaccinia virus.[1-4] Based upon knowledge of carbocyclic 3-deazaadenosine and the metabolism of nucleosides in general, we targeted three distinct classes of new compounds as potentially enhanced agents against this poxvirus: (1) CADO analogs with selected alterations at C-5'; (2) similar analogs based on 4'-thio-3-deazaadenosine; and (3) CADO prodrugs resulting from 5'-O-acylation. For this study, we eventually wanted to prepare 3-6 examples of the Class 1 and 2 compounds and 10 examples of the Class 3 compound, all in 25-100 mg quantities. Because of the effort required to prepare the parent compound for many of these analogs (CADO), we aimed at preparing ~6-10 new compounds during the first year.

Our initial efforts were directed toward the preparation of the Class 3 5'-O-acylated CADO prodrugs. Since we had only a small amount of CADO (~200 mg) in our repository, we also needed to devote a significant part of our efforts toward the preparation of more CADO. Following the preparation of these initial targets, we planned to redirect our efforts toward the preparation of the much more difficult Class 2 targets. Other future efforts would also be aimed at modifying our synthetic routes so they could be used in combinatorial approaches that would facilitate the optimization of these agents.

Unfortunately, our CADO analog synthesis efforts were less productive than we had planned, and we were able to supply Dr. Huggins with additional CADO and the 5'-O-benzoate of CADO. However, we were also able to Dr. Huggins with adenosine N^1-oxide and 22 adenosine N^1-oxide and benzyloxyadenosines analogs so that he could investigate their activities against variola and other poxviruses. These compounds were of interest to Dr. Huggins because of the significant activities against vaccinia virus that they had been shown in our laboratories.

Table 1. Target Compounds

Class 1



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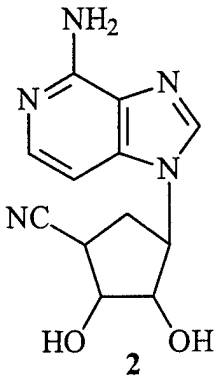

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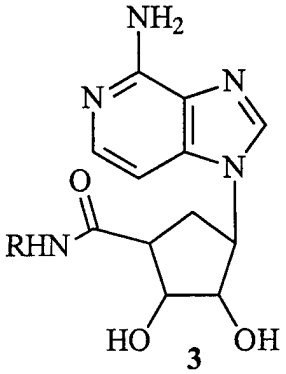

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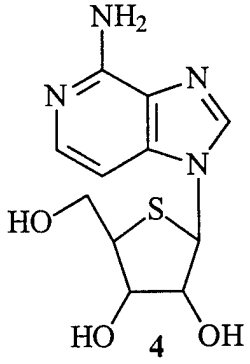
Class 2



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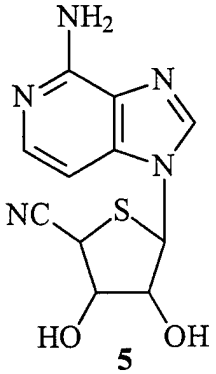

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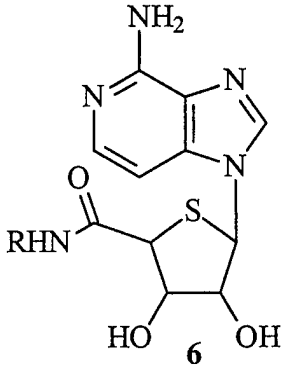

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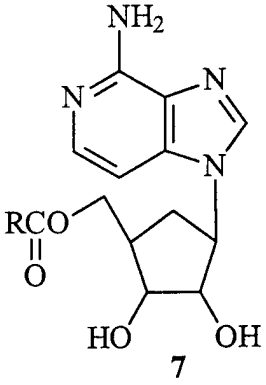
Class 3



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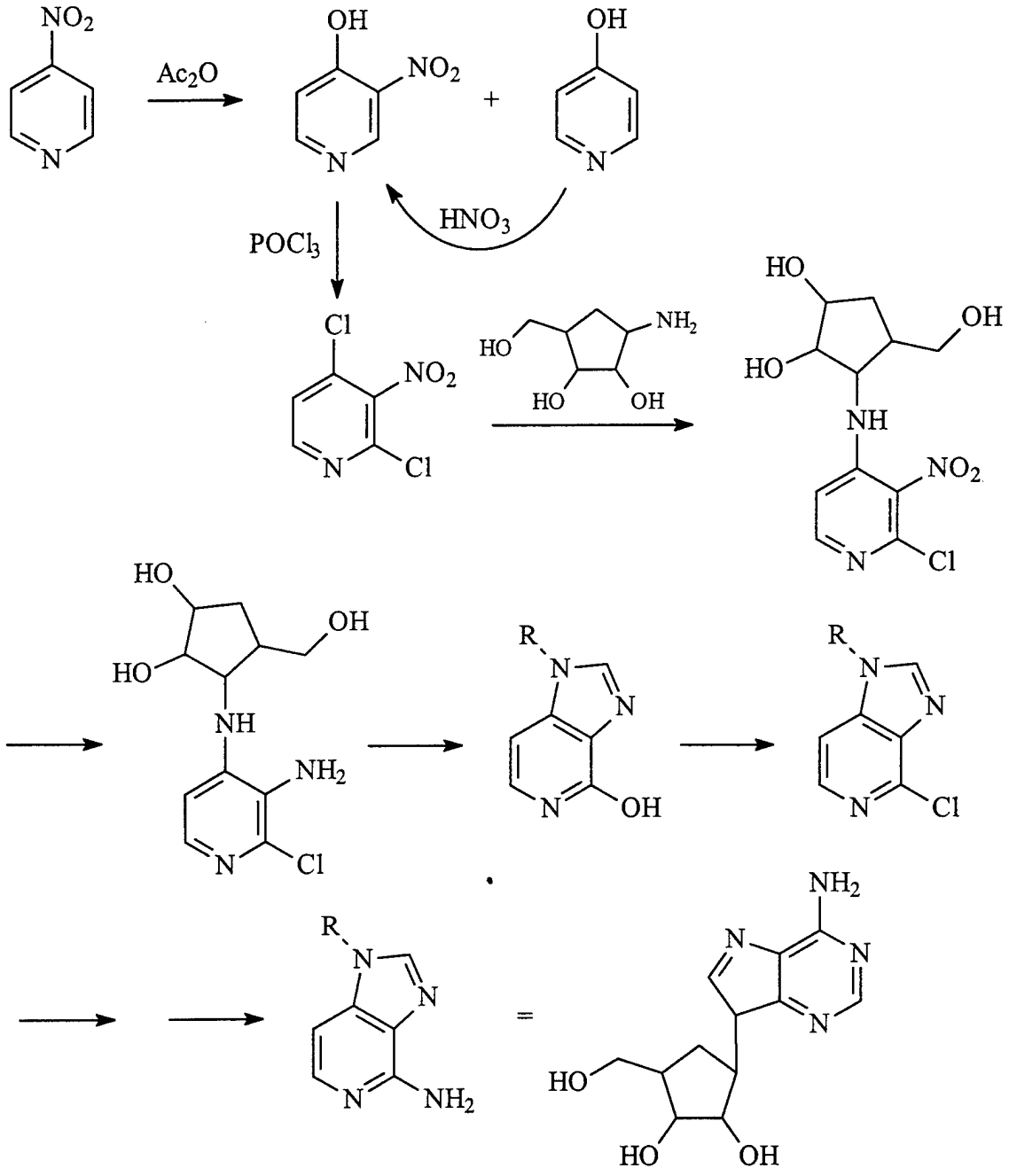
R = various alkyls or aryls

Body

5'-O-acylated CADO Targets

The early targets for our initial efforts to synthesize the 5'-O-acylated CADO prodrugs were the 5'-O-benzoate, acetate, and octanoate of CADO. Since these targets had not previously been reported, we reviewed the literature for 5'-O-acylated nucleosides or analogs and found general approaches that seemed appropriate for the synthesis of our targets: 5'-O-acylation of

2',3'-isopropylidene uridine followed by deprotection[5]; and the direct 5'-O-acylation of 9-β-D-arabinofuranosyladenine (Ara-A).[6-8] The enzymatic acylation of adenosine[9,10] also initially seemed like a possible approach. We chose not to pursue this approach because we could not find a convenient source for any of the reported enzymes (e.g., subtilisin, Candida antarctica lipases SP435-345A), because the possible alternative enzymes found commercially (e.g., Sigma) were generally expensive, and because we were uncertain that the successful enzymatic acylation of adenosine would also work with CADO. Therefore, of the two seemingly surer, more traditional synthetic approaches, we initially attempted the direct 5'-acylation of CADO, and we targeted the 5'-O-benzoate. We eventually tried treating CADO with benzoyl chloride in a variety of solvents (pyridine, DMF, DMAC, pyridine/DMF) and at various temperatures ranging from 0°C to room temperature. Unfortunately, from these attempts, we either saw no reaction due to solubility problems, or we obtained inseparable mixtures of products. Similar results were obtained with our attempts to prepare the 5'-O-acetate. Next, we shifted our efforts toward the benzoylation of 2',3'-IP-CADO, following the procedure used by Mizuno to synthesize ribonucleoside and diribonucleoside monophosphates.[7] We treated 2',3'-IP-CADO with benzoyl chloride and obtained the 5'-O-benzoylated product in good yield. Unfortunately, deprotection with 80% acetic acid gave the desired 5'-O-benzoate contaminated with a significant amount of benzoic acid, which we were unable to remove with repeated ether extractions, washes with $NaHCO_3$, or by repeated recrystallizations. We then repeated this approach except that we first purified the 2',3'-IP-CADO 5'-O-benzoate intermediate by washing with potassium carbonate (to remove any benzoic acid) and then chromatographed the mixture to remove unreacted 2',3'-IP-CADO. Deprotection with 80% acetic acid then gave the desired 5'-O-benzoate of CADO. Our subsequent attempts to do likewise for the preparation of the 5'-O-acetate were not successful, because the deprotection of the 2',3'-IP-CADO 5'-O-acetate was accompanied by a significant amount of deactylation. We were also unsuccessful in our recent attempt to prepare the 5'-O-octanoate, because 2',3'-IP-CADO was unreactive with octanoyl chloride, even after an extended period (3 days) at 20-30°C. Ongoing efforts on the 5'-O-acetate have been shifted to 5'-O-propionate which may be less susceptible to deacylation. We are currently investigating elevated temperatures in our attempts to synthesize the 5'-O-octanoate.

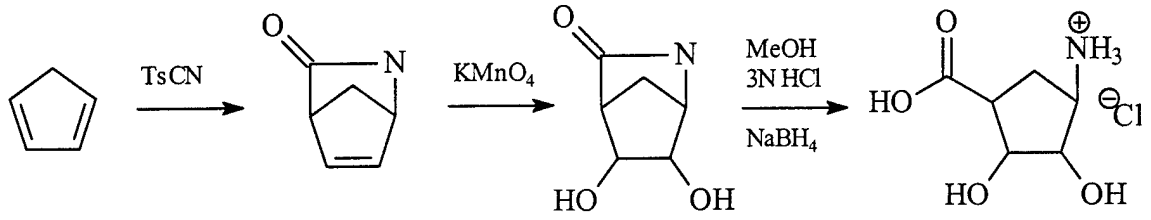


Preparation of CADO and its 6'-chloro precursor

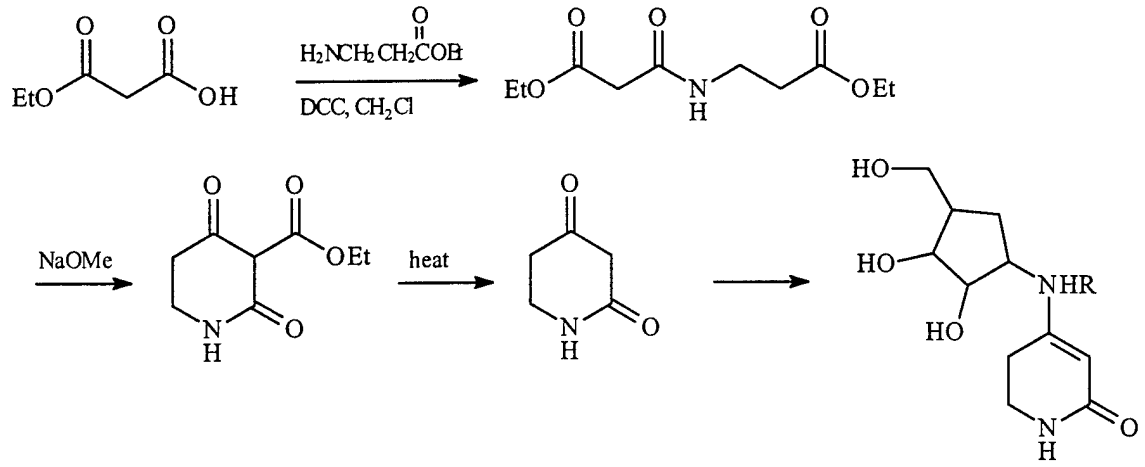
As previously mentioned, the synthesis of more CADO was required because CADO was the parent compound for this study and was also a precursor for many of the targets. CADO was eventually prepared according to the procedure by Montgomery et al.,[11] as shown below. 4-Nitropyridine was first heated with acetic anhydride to give a mixture containing 3-nitro-4-hydroxypyridine and 4-hydroxypyridine. The 4-hydroxypyridine was nitrated to 3-nitro-4-hydroxypyridine and then, both lots of 3-nitro-4-hydroxypyridine were combined and converted

to 2,4-dichloro-3-nitropyridine by treatment with phosphorus oxychloride. We then reacted 2,4-dichloro-3-nitropyridine with 2,3-dihydroxy-4-hydroxymethylcyclopentylamine to get the required aminosugar adduct. The nitro group of this adduct was then reduced with Raney nickel, and the resulting diaminopyridine was reacted with triethylformate and 12N HCl in dimethylacetamide to give the 6-chlorodeazapurine precursor to CADO. Treating this precursor with hydrazine followed by reduction with Raney nickel then gave CADO.

Since it was not commercially available, the aminosugar (2,3-dihydroxy-4-hydroxymethylcyclopentylamine) was also prepared in our labs by the procedure of Daluge and Vince.[12,13] Cyclopentadiene was first generated from dicyclopentadiene and then reacted with tosyl cyanide (prepared by treating an aqueous solution of the sodium salt of *p*-toluenesulfinic acid with cyanogen chloride). The double bond of the resulting bicyclic lactam was then oxidized with potassium permanganate to give the resulting desired *cis* diol. The lactam was then hydrolytically cleaved, converted to the corresponding aminoester intermediate with 3 N HCl and MeOH, and then reduced to 2,3-dihydroxy-4-hydroxymethylcyclopentylamine with $NaBH_4$.

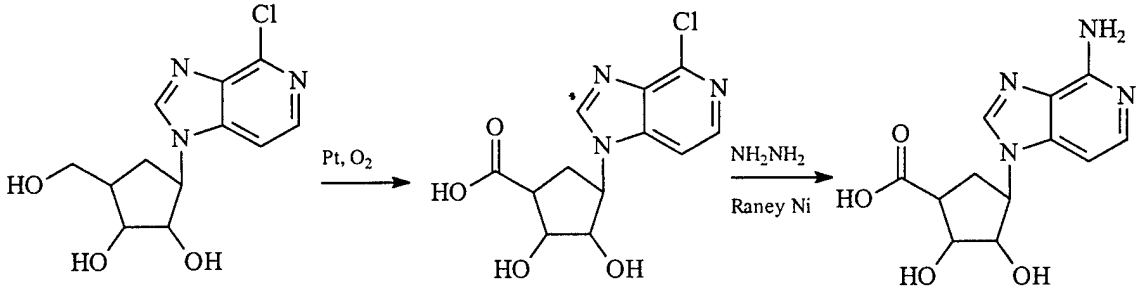


In the early stage of this work, we also investigated a possible alternate intermediate to replace 2,4-dichloro-3-nitropyridine in the Montgomery synthesis of CADO. This nitropyridine, while being easily synthesized, had also been found to cause severe rashes on many who had worked with it. Therefore, we were interested in an alternative to this compound and briefly looked at 2,4-dioxopiperidine[14,15] as a possible replacement. This dioxopiperidine was made by the scheme shown below in which the monoester of the ethyl ester of ß-alanine was added to the monoethyl malonate with dicyclohexylcarbodiimide. The resulting malonamide adduct was cyclized with sodium methoxide. The 3-ester function was then removed by heating in acetonitrile. We then found that both cyclopentylamine and our amino sugar (2,3-dihydroxy-4-hydroxymethylcyclopentylamine, as shown below) reacted at the 4-position to give the desired enamine adduct. However, because of time constraints and the pressing need for CADO in hand, this approach was not further investigated (N-functionalization of the 3-position, cyclization, conversion of the 2-oxo to 2-chloro to 2-amino, etc.,).



5'-COOH-CADO and congeners

All of these targets required 5'-carboxylic acid congener of CADO as their starting materials,[16,17] and thus, we attempted to prepare this compound according the method by Secrist et al.[16] According to this procedure, this compound should have been obtained in good yield by heating the 6-chloro precursor of CADO with reduced Pt oxide and O_2. Unfortunately, we were unsuccessful in repeated attempts to effect this conversion, consistently obtaining complex mixtures of products that were inseparable by chromatography. We were also unsuccessful in our attempts treating 2',3'-isopropylidene CADO with potassium permanganate, a method that, according to the literature, is effective for oxidizing 2'3'-isopropylidene adenosine.[18,19]



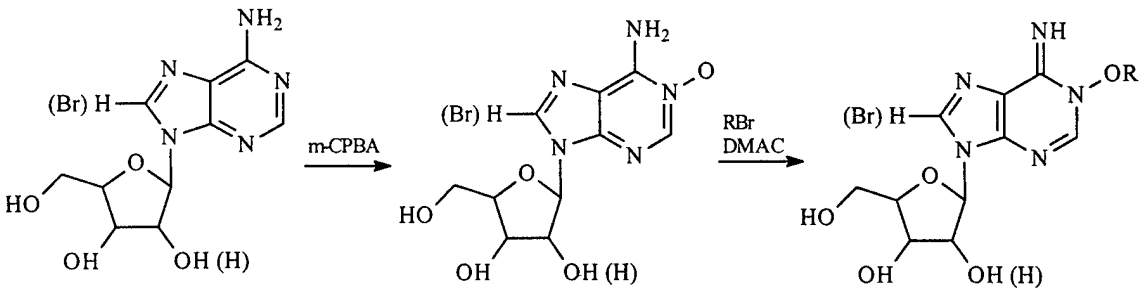
Benzyloxyadenosines

For this contract, we also supplied Dr. Huggins with adenosine N^1-oxide and the following 22 adenosine N^1-oxide and benzyloxyadenosines analogs so that he could investigate the activity of these compounds against variola and other poxviruses. We had previously prepared these compounds and found them to have activity against vaccinia virus as well as a number of other viruses.[20,21] Adenosine N^1-oxide was originally found to be a highly selective inhibitor of vaccinia virus replication in VERO cells using the CPE assay, and it also was found to have a relatively high therapeutic index (>300). Because of adenosine N^1-oxide's activity, a number of 1-benzyloxyadenosines were prepared and found to have comparable in vitro activity

Adenosine N^1-Oxide (SRI 4544)
N^1-(3-Methylbenzyloxy)adenosine (SRI 6767)
N^1-(2-Trifluoromethylbenzyloxy)adenosine (SRI 6768)
N^1-(4-Fluorobenzyloxy)adenosine (SRI 6769)
N^1-(4-Nitrobenzyloxy)adenosine (SRI 6886)
N^1-(2-Methylbenzyloxy)adenosine (SRI 6887)
N^1-(4-Cyanobenzyloxy)adenosine (SRI 6888)
N^1-(3-Methoxycarbonyllbenzyloxy)adenosine (SRI 6908)
N^1-(2-Cyanobenzyloxy)adenosine (SRI 6909)
N^1-(3-Cyanobenzyloxy)adenosine (SRI 6910)
N^1-(2-Methoxy-5-nitrobenzyloxy)adenosine (SRI 6911)
N^1-(3-Chlorobenzyloxy)adenosine (SRI 6916)
2-Deoxyadenosine, N^1-oxide (SRI 4305)
N^1-(2-nitrobenzyloxy)adenosine (SRI 6927)
N^1-(2,4-bis(trifluoromethyl)benzyloxy)adenosine (SRI 6928)
N^1-(3,5-bis(trifluoromethyl)benzyloxy)adenosine (SRI 6929)
N^1-(2,4-difluorobenzyloxy)adenosine (SRI 6987)
N^1-(3,4-difluorobenzyloxy)adenosine (SRI 6988)
N^1-(2-phenylethyloxy)adenosine (SRI 6989)
N^1-(3-methylbenzyloxy)-2'-doxyadenosine (SRI 6990)
N^1-(3-methoxylbenzyloxy)adenosine (SRI 7055)
N^1-(2-methylbenzyloxy)-2'-deoxyadenosine (SRI 7056)
N^1-(3-methylbenzyloxy)-8-bromo-adenosine (SRI 7191)

Adenosine-N^1-oxide was prepared by stirring adenosine and *m*-chloroperoxybenzoic acid in methanol at room temperature until thin-layer chromatography no longer showed the presence of starting material, usually after 15-20 h of stirring during which additional aliquots of MCPBA were added.[20]

The 1-benzyloxyadenosines were generally prepared by stirring a suspension of adenosine-N^1-oxide in molecular sieve (4Å) dried *N,N*-dimethylacetamide (DMAC) with the appropriate benzyl bromide. The 1-benzyloxyadenosines were isolated as their corresponding perchlorate salts, prepared by dissolving the product in H_2O and adding a warm aqueous solution of ammonium perchlorate.



Key Research Accomplishments

Our accomplishments include the preparation of more CADO (~10 g), the synthesis and delivery of the 5'-O-benzoate of CADO, and the delivery of adenosine N^1-oxide and 22 other adenosine N^1-oxide and 1-benzyloxyadenosine analogs for evaluation by Dr. Huggins' laboratory. Still ongoing are efforts to synthesize 1-2 other examples of our 5'-O-acylated CADO (Class 3) targets and 1-3 examples of the 5'-COOH-based (Class 1) targets.

Reportable Outcomes

The results of the evaluation of adenosine N^1-oxide and the 1-benzyloxyadenosine analogs will be presented at the upcoming ISAR in a poster under the following tentative title. (A copy of abstract is provided as Appendix A.) A more extensive manuscript is also being prepared.

> **Derivatives of Adenosine-N^1-Oxide Show Different Antiviral Activities Against Variola and Other Orthopoxviruses** R. O. Baker[1]*, C. D. Kwong[2], J. A. Secrist III[2], and J. W. Huggins[1]. [1]US Army Medical Research Institute of Infectious Diseases, Ft. Detrick, MD 21702, and [2]Southern Research Institute, Birmingham, AL, 35205.

Conclusions

Our primary goal of preparing CADO analogs as new potential agents against poxvirus was hindered by the short duration of the project and by unexpected difficulties in the isolation and purification of target compounds. Our overall productivity was also affected by our limited supply of CADO, which was also a key starting material for many of our targets. In the first half of this project, we synthesized a new supply of CADO and its 6-Cl precursor. In the latter half, we were able to synthesize and deliver our 5'-O-benzoate target. Unfortunately, we also found that the 5'-acetate was much more difficult to isolate because of what appeared to be an inherent instability. We are continuing with these efforts and have targeted the 5'-O-propionate with the hope that this acylated derivative will be more stable. We have been unsuccessful in our efforts to prepare the 5'-COOH-based derivatives because of our inability to reproduce the literature preparation of this compound or to apply the reported procedure for synthesizing the closely related 5'-COOH analog of adenosine. We are currently investigating other ways of approaching this compound.

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APPENDIX A

OCT-26

Activity of adenosine-N^1-oxide derivatives against variola and other orthopoxviruses. R. O. Baker[1]*, C. D. Kwong[2], J. A. Secrist III[2], and J. W. Huggins[1]. [1]US Army Medical Research Institute of Infectious Diseases, Ft. Detrick, MD, and [2]Southern Research Institute, Birmingham, AL.

Orthopoxvirus infections, including that of variola (VAR), the causative agent of smallpox, are of significant concern to both health and defense officials. In order to evaluate potential drugs useful in combating occurrences of orthopoxvirus infections, we have examined the sensitivity of four isolates of VAR, as well as monkeypox (MPX), cowpox (CPX) and vaccinia (VAC) viruses to a series of 11 drugs (chemically-modified derivatives of adenosine-N^1-oxide (ANO), a drug known to have activity against VAC), believed to be inhibitors of viral protein synthesis. We have employed *in vitro* assays using live virus handled under biosafety level 4 containment in a 96-well plate format to determine drug efficacy and toxicity in several monkey and human cell lines. These assays use neutral red uptake to determine cell viability after infection and/or drug treatment. All drugs tested showed low cytotoxicity (TC_{50} = 114 - ≥370 μM). Derivative compounds showed varying levels of activity, falling into three main groups. Six drug derivatives were highly active, with IC_{50} values for VAR between 0.07 and 1.0 μM, and between 0.37 and 6.7 μM for the other viral species. These six drugs had therapeutic indices from 28 to more than 3300. One drug showed intermediate activity, with an IC_{50} ranging from 1.1 to 5.6 μM for isolates of VAR, and 21.8 to 38.9 μM for the other viral species. The remaining four derivatives showed the least activity, with IC_{50} values for VAR from 2.8 to 101 μM, and 94 to ≥270 μM for other viruses. There exist significant differences in sensitivity to some of the drugs tested; ≥100-fold among VAR, MPX, CPX and VAC viruses, and up to 20-fold among the four VAR isolates. These drugs also showed cell line-dependent variations in IC_{50} values. We conclude from these data that at least several derivatives of ANO are excellent candidates for further development as anti-pox agents.